IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: CERBIOS-PHARMA S.A.

Serial No. 09/815,533

Filed : March 16th, 2001

Title: "A METHOD FOR THE PRODUCTION OF PHARMACEUTICALLY ACTIVE RECOMBINANT PROTEINS"

DECLARATION UNDER CFR1.132

I, Antonio Baici, being duly sworn depose and say that:

1. I am an Italian citizen residing in Zurich, Switzerland

2. I am familiar with the English language.

I further declare that:

A) University attended

I graduated in Chemistry at the University of Trieste (Italy) in the academic year 1970. I am author of 102 scientific publications (see the enclosed list).

B) Previous professional experience

From 1972 to 1978 I was employed at the Swiss Federal Institute of Technology in Zurich. I was involved in research on thermodynamics, kinetics, binding and allosteric interactions of dehydrogenases and other enzymes. I was also concerned with conformational properties, spectroscopy and nuclear magnetic resonance of synthetic peptides. From 1978 to 2001 I was head of a research group at the University Hospital of Zurich, Switzerland, Department of Rheumatology.

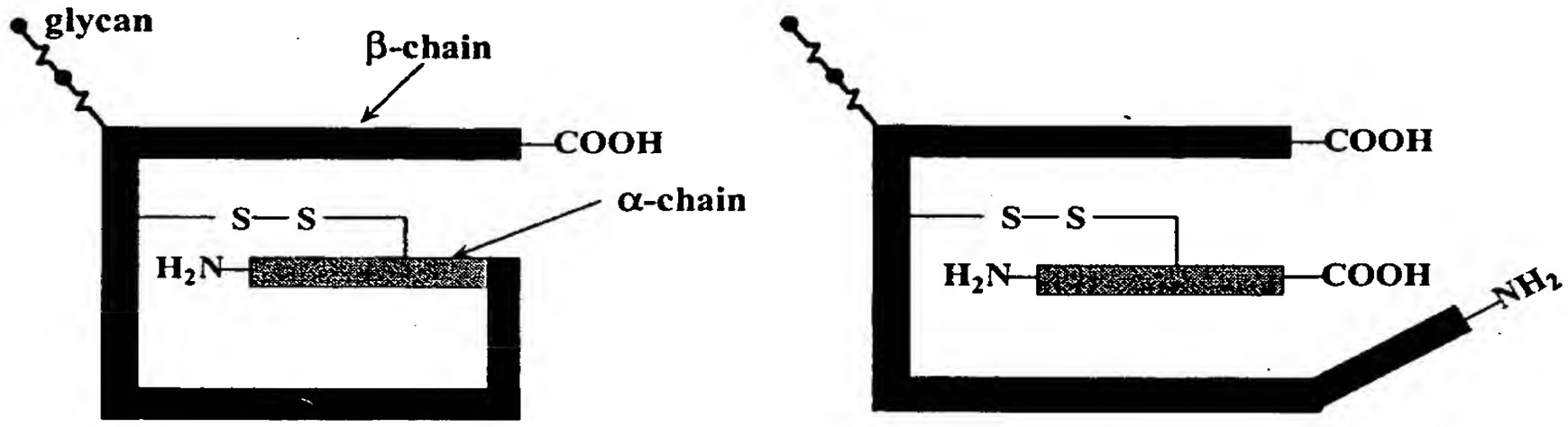
C) Present position

Since April 2001 I am working in quality of Professor of Biochemistry at the Medical and Natural Sciences Faculties of the University of Zurich, where I am also leader of a research group. The main topic of my research activity since 1978 is the characterization of proteolytic enzymes, with emphasis on the significance of proteolysis for tissue destruction in tumor invasion and rheumatic diseases. The present major research topic concerns the analysis of a pathogenic mechanism in osteoarthritis based on phenotypic changes on the part of the chondrocytes. Other details can be found in my curriculum vitae.

I have 31 years of professional experience in enzymology and, in particular, 25 years of specific expertise in the field of proteolytic enzymes.

3. On the basis of the personal experience deriving from my research activity I declare what follows:

A fundamental property of the present invention is represented by the proteolytic activation of human recombinant single-chain urokinase (sc-uPA) in the mentioned cell cultures in the presence of butyrate or of other short chain alkanoic acids and their derivatives, as listed in the specification at page 5, lines 10-14. Figure 1 schematically illustrates the structures of inactive sc-uPA and of active two-chain urokinase (tc-uPA).



sc-uPA **tc-uPA**

Figure 1. Schematic structure of human high molecular weight (HMW) urokinase. Left: enzymatically inactive single-chain urokinase (sc-uPA); right: enzymatically active two-chain urokinase (tc-uPA). Upon reduction of the disulfide bridge linking the α and the β chain, the protein migrates as two bands in a polyacrylamide gel (Figure 2).

As shown in Figure 2, treatment of the cell cultures for a time greater than 24 hours results in the almost complete conversion of sc-uPA into tc-uPA, i.e. the active form of this proteolytic enzyme (reaching a value higher than 95%, as reported on page 9 of the specification). Densitometric analysis of a Western blot run under reducing conditions, and stained with polyclonal antibodies specific for urokinase, demonstrates in a straightforward way that tc-uPA is the major product of urokinase found in the supernatants of butyrate-treated cell cultures. The activation process occurs through proteolysis of the urokinase precursor under mild conditions that provide the fully active enzyme at a high yield.

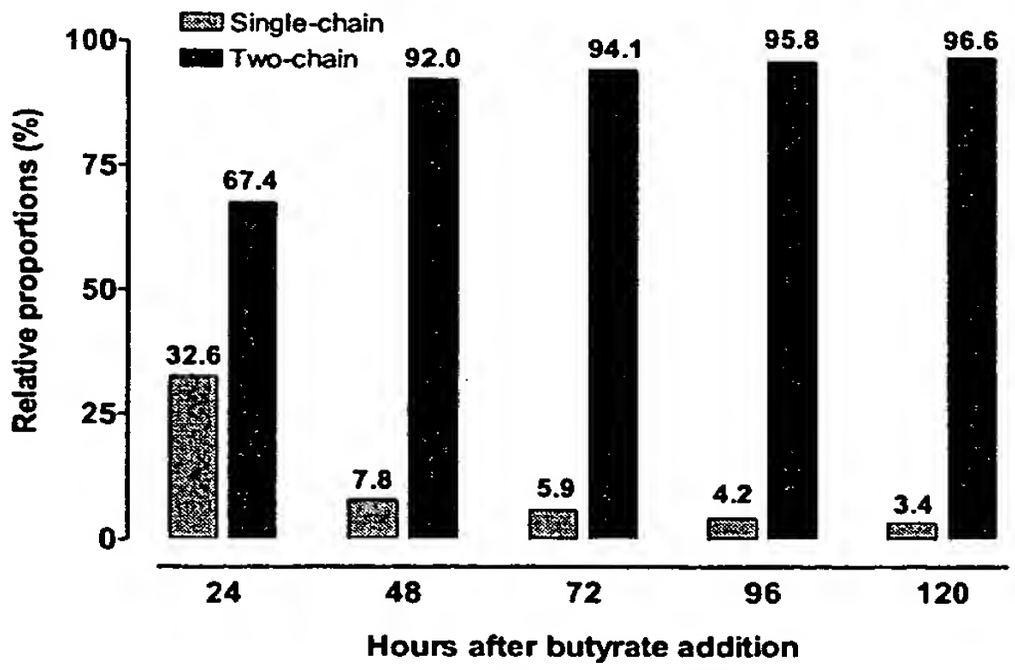


Figure 2. Densitometric diagram of the urokinase bands resulting from Western blot analysis of the culture medium during sodium butyrate treatment. An equal volume of samples was applied to five lanes of a sodium dodecyl sulphate polyacrylamide gel, and electrophoresis was run under reducing conditions. Following blotting to an Immobilon® membrane, staining was performed with antibodies against urokinase. The bar diagram shows the relative proportions of the bands within a lane expressed as the percentage of sc-uPA and tc-uPA (actual percentages shown on top of the bars).

On the basis of these observations, I can assure that the assertions relative to the specific points described herein, and contained in the patent application, are true and supported by sound experimental evidence.

The essence of the present invention resides in the considerable increase of proteolytic activity in the supernatant of a eucaryotic cell line culture after addition of sodium butyrate. The zymogram run with supernatants of butyrate-treated cell cultures clearly show a large increase of gelatinolytic activity with respect to cell cultures not treated with butyrate (Figure 3). More precisely, the supernatant of the butyrate-treated cells clearly shows an increase in gelatinolytic activity that is far superior to that of untreated cells. This is clearly shown in the experiment by incubating the zymogram at 37 °C (Figure 3). In a further experiment, addition of the chelating agent EDTA demonstrated the complete inhibition of gelatinolytic activity. This pattern of activity fully agrees with the increased secretion of a gelatinolytic enzyme, during incubation of the cells in the presence of butyrate. Gelatinases are proteolytic enzymes that need the zinc ion as cofactor for their activity, and belong to the class of matrix metalloproteinases (MMPs). The inhibition by EDTA, which subtracts zinc by chelation, is thus diagnostic for the presence of a gelatinolytic MMP. The molecular mass of about 92 kDa found in the zymogram corresponds to the known molecular mass of mammalian gelatinase B (MMP-9) (Okada et al., 1995). This enzyme plays a central role among the MMPs in physiological processes such as embryogenesis, morphogenesis and development but also in pathological situations. Once gelatinase and other MMPs are synthesized and released by cells as inactive zymogens, enzyme latency and activation represent a pivotal point of control. Several enzymes, including serine peptidases and various MMPs, are activated in the extracellular space in a cascade of proteolytic events in which an enzyme is activated by another preceding it in the cascade (Nagase, 1997; Van den Steen et al., 2001).

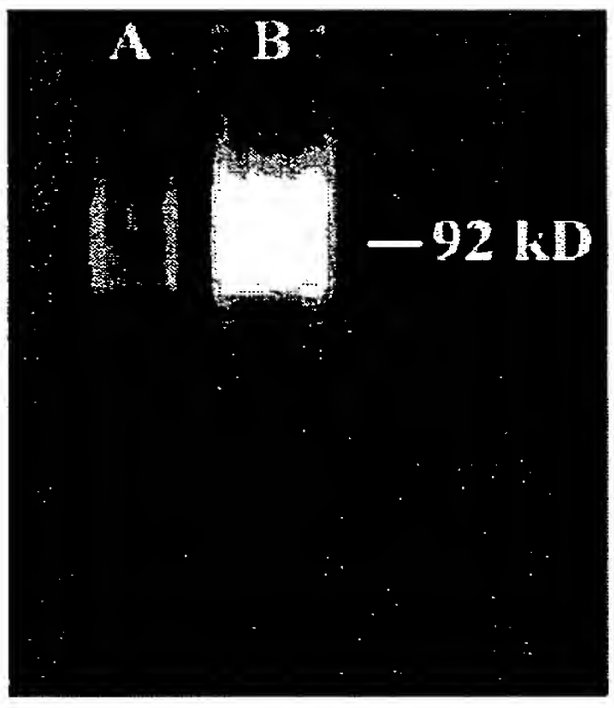


Figure 3. Zymogram of cell culture supernatants. Lane A: untreated; lane B: after five days of treatment with sodium butyrate. The cell density of the cultures was the same in butyrate-treated and in untreated samples.

In conclusion, the proven enhancement of the activity of the proteases in the presence of butyrate in the culture medium of the recombinant urokinase-producing CHO cell line is in line with the concept of an increased conversion of sc-uPA to active tc-uPA when the incubation time is greater than 24 hours. Thus, based on the above observations, the process described in the present invention supports the claim of a general activation mechanism of zymogens in the extracellular space of cell cultures treated with butyrate.

4. I finally declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that such willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the applications or any patents or re-examination certificate issued thereon.

Date: April 15, 2003 Antonio Rawa

References

Nagase, H. (1997). Activation mechanisms of matrix metalloproteinases. Biol. Chem. *378*, 151-160.

Okada, Y., Naka, K., Kawamura, K., Matsumoto, T., Nakanishi, I., Fujimoto, N., Sato, H., and Seiki, M. (1995). Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase = gelatinase B) in osteoclasts: implications for bone resorption. Lab. Invest. *72*, 311-322.

Van den Steen, P. E., Opdenakker, G., Wormald, M. R., Dwek, R. A., and Rudd, P. M. (2001). Matrix remodelling enzymes, the protease cascade and glycosylation. Biochim. Biophys. Acta *1528*, 61-73.